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(54) Title: APPLICATION OF REDUCING SULPHUR COMPOUNDS DURING ENZYMATIC PREPARATION OF β-LACTAM COMPOUNDS

(57) Abstract

Disclosed is a method to improve the quality of enzymatic hydrolysis or synthesis of the substituted β-lactam compounds with respect to enzyme productivity, product quality and process streamlining (prevention of the formation deposits) by the addition of reducing sulphur compounds, such as sodium sulphite or sodium metabisulphite.

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Application of reducing sulphur compounds during enzymatic preparation of β-lactam compounds

The present invention relates to an amended preparation of β-lactam compounds.

β-Lactam antibiotics constitute the most important group of antibiotic compounds, with a long history of clinical use. Among this group, the prominent ones are the penicillins and cephalosporins. These compounds are naturally produced by the filamentous fungi *Penicillium chrysogenum* and *Acremonium chrysogenum*, respectively.

Nowadays, β-lactam antibiotics, such as penicillin and cephalosporin antibiotics, play an important role in medicine. This class of antibiotics comprises a great variety of compounds, all having their own activity profile. In general, β-lactam antibiotics consist of a nucleus, the so-called β-lactam nucleus, which is linked through its primary amino group to the so-called side chain via an amide bond. The term β-lactam nucleus includes amino-β-lactams such as 6-amino penicillanic acid (6-APA), 7-amino-cephalosporanic acid (7-ACA), 7-amino-3-chloroceph-3-em-4-carboxylic acid (7-ACCA), 7-aminodesacetyl-cephalosporanic acid (7-ADAC), and 7-aminodesacetoxy-cephalosporanic acid (7-ADCA).

The term β-lactam antibiotics as used herein includes fermentation products, such as penicillin G, penicillin V, cephalosporin C, isopenicillin N, intermediate products such as adipyl-7-aminodesacetoxycephalosporanic acid (adipyl-7-ADCA), phenylacetamido-desacetoxy cephalosporanic acid (PADCA), adipyl-7-aminocephalosporanic acid (adipyl-7-ACA), adipyl-7-aminodesacetylcephalosporanic acid (adipyl-7-ADAC), 3-carboxyethylthiopropionyl-7-aminodesacetoxycephalosporanic acid, 2-carboxylethylthioacetyl-7-aminodesacetoxy-cephalosporanic acid and 3-carboxyethylthiopropionyl-7-aminodesacetoxycephalosporanic acid, and semi-synthetic

products such as Ampicillin, Amoxicillin, Cephalexin, Cefaclor and Cefadroxil and Cephradine.

There has been a lot of interest for the so called enzymatic semi-synthetic routes to β-lactam antibiotics. These routes lack many of the disadvantages of the conventional synthetic methods for preparing β-lactam antibiotics. The enzymatic catalyzed reactions are highly selective, thus the production of many by-products, and the effluent and purification problems, which result therefrom, are avoided. Furthermore, enzymatic processes can be performed in aqueous environment.

The semi-synthetic routes mostly start from fermentation products such as isopenicillin N, penicillin G, penicillin V, Cephalosporin C, adipyl-7-ADCA, adipyl-7-ACA, adipyl-7-ADAC and adipyl-6-APA, which are enzymatically converted to a β-lactam nucleus, for instance in a manner as has been disclosed in K. Matsumoto, Bioprocess. Technol., 16, (1993), 67-88, J.G. Shewale & H. Sivaraman, Process Biochemistry, August 1989, 146-154, T.A. Savidge, Biotechnology of Industrial Antibiotics (Ed. E.J. Vandamme) Marcel Dekker, New York, 1984, or J.G. Shewale et al., Process Biochemistry International, June 1990, 97-103. The obtained β-lactam nucleus is subsequently converted to the desired antibiotic by coupling to a suitable side chain, as has been described in *inter alia* EP-A-0 339 751, JP-A-53 005 185 and CH-A-640 240. By making different combinations of side chains and β-lactam nuclei, a variety of penicillin and cephalosporin antibiotics may be obtained. For example, a D-(-)-phenylglycine side chain may be attached to a 6-aminopenicillanic acid (6-APA) nucleus to yield Ampicillin, or to a 7-aminodesacetoxycephalosporanic acid (7-ADCA) nucleus to yield Cephalexin.

The known enzymatic methods for preparing β-lactam antibiotics all involve the preparation of a β-lactam nucleus and the subsequent coupling thereof to a suitable side chain. References for enzymatic synthesis are: T.A. Savidge, Biotechnology of Industrial Antiobiotics (Ed. E.J. Vandamme) Marcel Dekker, New York 1984,J.G. Shewale et al., Process Biochemistry International, June 1990 97-103, E.J.

Vandamme, Advances in Applied Microbiology, 21, (1977), 89-123 and E.J. Vandamme, Enzyme Microb. Technol., 5, (1983), 403-416. In addition, new routes have been disclosed, which show the direct fermentative production of 7-ADCA, 7-ADAC and 7-ACA, in EP-A-0 540 210, WO-A-93/08287, WO-A-95/04148 and WO-A-95/04149.

During the preparation and enzymatic conversion of industrially produced β-lactam compounds (with a purity of >95%) minute amounts of sulphur (typically: 0.01-0.05 w/w %) are found to deposit. When using the fill-and-draw process mode, the sulphur will accumulate on the sieving device and after some time start clogging and thus delaying the separation of enzyme and reaction product.

A simple method to diminish the problem is to remove sulphur from the freshly prepared solution comprising the starting material (by e.g. filtration), prior to adding it to the enzyme. In this way the formation of sulphur during the enzymatic conversion will not be prevented.

Recrystallization of the starting material (e.g.: from methanol) will also diminish the amount of coloured compounds in the solution prior to the enzymatic conversion and, to some extent, in the final product, too. A reduction to zero of the amount of sulphur formed per cycle is however not achieved in this way.

Although improvements can be achieved by the recrystallization procedure, from an economical point of view recrystallization of the starting β-lactam compound is not very attractive, mainly by the considerable loss of the starting β-lactam compound in this process step.

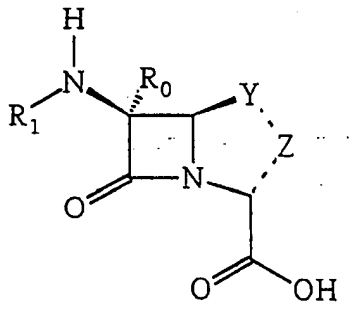
An alternative to recrystallization to remove the apparent enzyme-inactivating agent was found to be a pre-treatment of the PADCA solution with activated carbon. Combination of the effects of the carbon treatment and the filtration of solution comprising the starting material prior to the enzymic hydrolysis proved to yield both an improvement of enzyme stability and a reduction of the amount of sulphur deposited during a conversion cycle (cf. Example 1). The quality of the final product after the isolation procedure did not improve much (cf. Example 4).

As major drawback of the carbon treatment should be mentioned that the removal of the carbon is rather cost-intensive (also due to the inevitable losses of the starting β-lactam compound in this process step), as is the disposal of the carbon with respect to the environmental aspects.

A method should therefor be found to remove the small amounts of sulphur present in the starting β-lactam solution without the drawbacks of the methods described above.

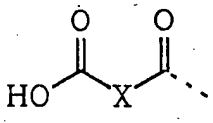
Surprisingly, it was found that by the addition of a reducing sulphur compound not only elementary sulphur was removed, but that also both the β-lactam compounds produced displayed a dramatic quality improvement with regard to colour and the enzyme stability increased.

The present invention provides therefore a process for the preparation of a β-lactam antibiotic with the general formula (I)



(I)

wherein R_1 is either hydrogen or



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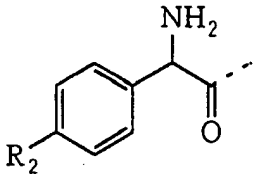

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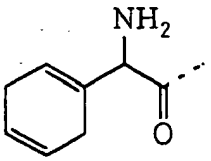


with

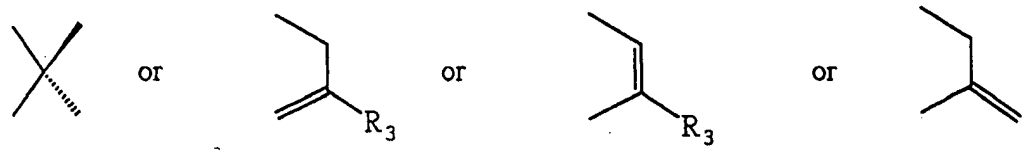
X is $(CH_2)_m$-A-$(CH_2)_n$, wherein m and n are the same or different and are chosen from the group of integers 0, 1, 2, 3 or 4, and A is CH=CH, C≡C, CHB, C=O, optionally substituted nitrogen, oxygen, sulphur or an optionally oxidized form of sulphur, and B is hydrogen, halogen, hydroxy, C_{1-3} alkoxy, or optionally substituted methyl, R_2 is hydrogen or hydroxy;

and wherein

R_0 is hydrogen or C_{1-3} alkoxy;

Y is CH_2, oxygen, sulphur, or an oxidized form of sulphur;

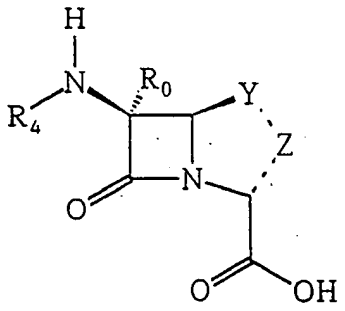
Z is



wherein R_3 is hydrogen, hydroxy, halogen, C_{1-3} alkoxy, optionally substituted, optionally containing one or more heteroatoms, saturated or unsaturated, branched or straight C_{1-5} alkyl, optionally substituted, optionally containing one or more heteroatoms, C_{5-8} cycloalkyl, optionally substituted aryl or heteroaryl, or optionally substituted benzyl,

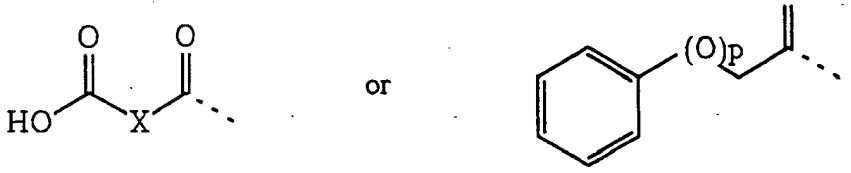
or a salt thereof,

by reacting a compound with the formula I'



(I')

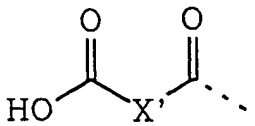
with R_4 is either



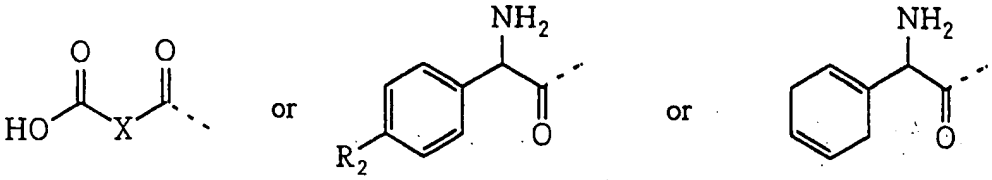
with X as defined above, and p = 0 or 1,

when R_1 is hydrogen, and each of R_0, Y and Z have corresponding meanings in β-lactams with formula I and I', with a dicarboxylate acylase, or a penicillin acylase respectively;

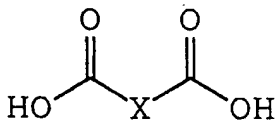
or when R_4 is H, or



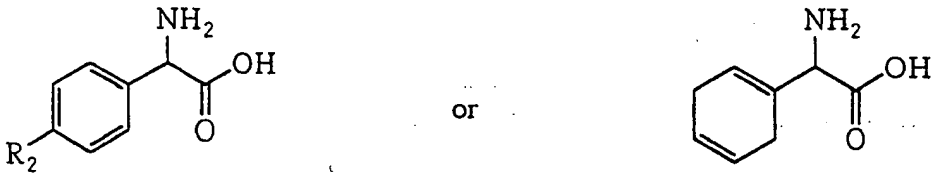
with X' is defined as X but different from the same,
when R_1 is



respectively, and each of R_0, Y and Z have corresponding meanings in β-lactams with formula I and I',
with a precursor for a side chain of the formula



or



respectively, or an amide or ester thereof
and a dicarboxylate acylase or a penicillin acylase, respectively if R_4 is hydrogen, and a penicillin acylase and dicarboxylate acylase of R_4 is not hydrogen,
characterized by the application of a sulphur reducing compound.

Preferably, a reducing sulphur compound, selected from the group consisting of sulphite, metabisulphite, dithiothreitol, β-mercaptoethanol and dithiotrite, has been applied.

Brief description of the figures

Figure 1
Absorbance at 425 nm of a 3'-desacetoxycephalosporin G solution without additive, with bisuphite, and with carbon as function of time.

Figure 2

Turbidity of a 3'-desacetoxycephalosporin G solution without additive, with bisulphite, and with carbon as function of time.

Figure 3
Absorbance at 425 nm of a 7-ADCA solution without additive, with bisulphite, and with carbon as function of time.

Figure 4
Turbidity of a 7-ADCA solution without additive, with bisulphite, and with carbon as function of time.

Figure 5
Penicillin acylase stability as a function of the amount of sodium metabisulphite added.

Detailed description of the invention

For the enzymatic conversion of β-lactam compounds a number of key factors determine the economic quality of said conversion, for instance quality of final product 7-ADCA, enzyme costs and yield.

To achieve an economically attractive process, these key factors have to be optimized. A closer look will place the current invention into perspective.

First of all, the quality of final product (among others expressed as colour, i.e.: absorption at 425 nm).

The quality of for instance 7-ADCA as final product is to a large extent determined by the quality of the recovery of 7-ADCA from the hydrolysis reaction mixture. By the way, in case of 7-ADCA production the recovery procedure consists of bringing the pH below 1, extraction of phenylacetic acid or adipic acid with an alcohol, phase separation, crystallization of 7-ADCA by bringing the aqueous phase to pH 4-5, filtration, washing and drying. However, of course also the composition of the reaction mixture at the end of the hydrolytic process is important for the quality of the end product. It will be clear that avoiding the formation of by-products during the enzymatic hydrolysis will make any

recovery method more simple and more effective. An obvious way to achieve low by-product formation during hydrolysis is to minimize the reaction time and to restrict to mild reaction conditions with respect to e.g. temperature and pH.

Secondly, enzyme costs (loss of enzymatic activity during the production of for instance 7-ADCA) can be increased by thermal, microbial, proteolytical or chemical inactivation of the immobilized enzyme during the reaction cycle. Part of the loss is caused by the inherent, restricted stability of the catalyst, but the reaction conditions will affect the stability very strongly. Some of the main factors to enhance enzyme stability are:

- minimize reaction time, i.e.: avoid time consuming delays in non-productive periods
- minimize the amount of enzyme present in reactor
- avoid/remove substances, capable in spoiling enzyme activity
- add enzyme stabilizing agents.

Thirdly, yield (defined as moles produced endproduct recovered per mole starting material).

The maximal achievable yield will be determined by e.g. degradation of both starting material and endproduct during hydrolysis.

Both starting material and endproduct of enzymatic reaction of β-lactam compounds are quite stable substances; the mechanism of the reaction leading towards a sulphur-producing degradation or to the formation of coloured compounds, is not well-understood.

Surprisingly it has been found that a way of removing elementary sulphur is to dissolve the deposit by adding a reducing sulphur-compound (such as sulphite, metabisulphite, dithiothreitol, β-mercaptoethanol, dithionite, etc.). Within minutes even small amounts of reducing sulphur-compounds are capable of bringing this about, and some overdosage results in the absence of deposit-formation over an extended period. Preferably, amounts in the range of 0.02-1% by weight sulphur compound compared to reaction mixture are added, more preferably in an amount of 0.05-0.2 weight %.

Surprisingly it was found that by the addition of a reducing sulphur-compound:

- reductions of the colour expressed as the optical absorption at 425 nm) of both PADCA and 7-ADCA solutions are caused, albeit in a much slower rate than the dissolution of sulphur

- enzyme stability is increased, more effective than by using purified (recrystallized from methanol) or carbon treated PADCA-solutions.

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11, also known as penicillin amidase) catalyses both the acylation of a β-lactam nucleus (6-APA, 7-ACA, 7-ACCA, 7-ADAC and 7-ADCA) with an optionally activated side chain (phenylglycine, 4-hydroxyphenylglycine, dihydrophenylglycine, adipic acid, and the like), with the formation of β-lactam antibiotics and the enzymes, as isolated from *E.coli*, *A.faecalis*, *K.citrophilla*, *B. megaterium* and others are also capable of hydrolysing N-substituted β-lactams into a β-lactam nucleus such as 6-APA, 7-ACA, 7-ADCA, etc. and the side chain. 7-ADCA is widely used as an intermediate in the production of e.g. cephalexine; 6-APA is widely used as an intermediate in the production of e.g. amoxicillin and ampicillin.

Penicillin acylase is most commonly applied in immobilized form to enable reuse of the enzyme. Although it is in principle possible to perform the deacylation process in a continuous mode, the batch mode is considered to be the most favourable method of operation in the production of 6-APA and 7-ADCA from penicillin G or penicillin V and from N-substituted cephalosporins (as for instance adipyl-7-ADCA), respectively. In this mode, each new batch of penicillin G, penicillin V or phenylacetyl-ADCA (PADCA) solution is added to the immobilized enzyme, residing in the reactor; at the end of the deacylating process the reaction product is withdrawn from the reactor via a sieving device to keep the enzyme particles inside the reactor, ready for the next cycle. This batch mode is often referred to as a "fill-and-draw" system.

A suitable dicarboxylate acylase with which the *N*-substituted β-lactam is contacted in a method according to the invention is an enzyme that may be isolated from various naturally occurring micro-organisms, such as fungi and bacteria. Such micro-organisms can be screened for enzymes with the desired dicarboxylic acid specificity by monitoring the hydrolysis of suitable substrates. Such suitable substrates may be e.g. chromophores such as succinyl-, glutaryl- or adipyl-p-nitroanilide. Also, the hydrolysis of the corresponding *N*-substituted β-lactams may be used for identifying the required enzymes. It was found that the optimum pH range for these enzymes lies between about 6, preferably about 7, and about 9, preferably about 8.

Organisms that have been found to produce dicarboxyl acylase are *Alcaligenes*, *Arthrobacter*, *Achromobacter*, *Aspergillus*, *Acinetobacter*, *Bacillus* and *Pseudomonas* species. More in particular, the following species produce highly suitable dicarboxylate acylases: *Achromobacter xylosooxidans*, *Arthrobacter viscosis*, *Arthrobacter* CA128, *Bacillus* CA78, *Bacillus megaterium* ATCC53667, *Bacillus cereus*, *Bacillus laterosporus* J1, *Paecilomyces* C2106, *Pseudomonas diminuta* sp N176, *Pseudomonas diminuta* sp V22, *Pseudomonas paucimobilis*, *Pseudomonas diminuta* BL072, *Pseudomonas* strain C427, *Pseudomonas* sp SE83, *Pseudomonas* sp SE495, *Pseudomonas ovalis* ATCC950, *Comamonas* sp SY77, *Pseudomonas* GK 16, *Pseudomonas* SY-77-1, *Pseudomonas* sp A14, *Pseudomonas vesicularis* B965, *Pseudomonas syringae*, *Ps putida* ATCC17390, *Ps aeroginosa* NCTC 10701, *Proteas vulgaris* ATCC9634, *Ps fragi* DSM3881, and *B. subtilus* IFO3025.

The dicarboxylate acylase may be obtained from the microorganism by which it is produced in any suitable manner, for example as is described for the *Pseudomonas* sp SE83 strain in US-A-4,774,179. Also, the genes for e.g. SE83 or SY77 dicarboxylate acylases may be expressed in a different suitable host, such as *E.coli*, as has been reported by Matsuda et al. in J. Bacteriology, 169, (1987), 5818-5820 for the SE83 strain, and in US-A-5,457,032 for the SY77 strain.

The enzymes isolated from the above sources are often referred to as glutaryl acylases. However, the side chain specificity of the enzymes is not limited to the glutaryl side chain, but comprises also smaller and larger dicarboxyl side chains. Some of the dicarboxylate acylases also express gamma-glutamyl transpeptidase activity and are therefore sometimes classified as gamma-glutamyl transpeptidases.

A suitable penicillin acylase with which the *N*-substituted β-lactam is contacted in a method according to an aspect of the invention is an enzyme that may be isolated from various naturally occurring micro organisms, such as fungi and bacteria. Such micro organisms can be screened for enzymes with the desired specifity in a monitoring test analogous to the one described for the dicarboxylate acylase. Of these enzymes it was found that the optimum pH lies between about 4, preferably about 5, and about 7, preferably about 6.

Organisms that have been found to produce penicillin acylase are, for example, *Acetobacter*, *Aeromonas*, *Alcaligenes*, *Aphanocladium*, *Bacillus* sp., *Cephalosporium*, *Escherichia*, *Flavobacterium*, *Kluyvera*, *Mycoplana*, *Protaminobacter*, *Pseudomonas* or *Xanthomonas* species. Enzymes derived from *Acetobacter pasteurioanum*, *Alcaligenes faecalis*, *Bacillus megaterium*, *Escherichia coli* and *Xanthomonas citrii* have particularly proven to be succesful in a method according to the invention. In the literature, penicillin acylases have also been referred to as penicillin amidases.

The dicarboxylate acylase and penicillin acylase may be used as free enzymes, but also in any suitable immobilized form, for instance as has been described in EP-A-0 222 462 and WO-A-97/04086. It is possible to perform a method according to the invention wherein both enzymes are immobilized on one carrier or wherein the enzymes are immobilized on different carriers. In addition, it is possible to use functional equivalents of one or both of the enzymes, wherein for instance properties of the enzymes, such as pH dependence, thermostability or specific activity may be affected by chemical modification or cross-linking,

without significant consequences for the activity, in kind, not in amount, of the enzymes in a method according to the invention. Also, functional equivalents such as mutants or other derivatives, obtained by classic means or via recombinant DNA methodology, biologically active parts or hybrids of the enzymes may be used. In some cases, modification, chemical or otherwise, may be beneficial in a method according to the invention, as is part of the standard knowledge of the person skilled in the art.

The precursor for a side chain of the β-lactam antibiotic to be prepared in a method according to the invention may be any compound that is recognized by the above defined penicillin acylases and leads to a product of the class of β-lactam antibiotics. Preferably, the substrate is chosen from the group of D-(-)-phenylglycine, D-(-)-4-hydroxyphenylglycine, D-(-)-2,5-dihydrophenylglycine, 2-thienylacetic acid, 2-(2-amino-4-thiazolyl)-2-methoxy-iminoacetic acid, α-(4-pyridylthio)acetic acid, 3-thiophenemalonic acid, or 2-cyanoacetic acid, and derivatives thereof, as these substrates lead to β-lactam antibiotics having the most advantageous activity profile. Suitable derivatives of these substrates are esters and amides, wherein the side chain molecule is connected to a C_1-C_3 alkyl group through an ester or amide linkage. Preferably, the enzymes are added together to the *N*-substituted β-lactam and the precursor for the side chain.

From the fact that during enzymatic hydrolysis no extra colour formation is observed, it is obvious that the quality of the final product is improved considerably without affecting the over-all yield. Indirectly, the inhibition of colour-formation during the hydrolysis implies that a more cost-effective process is possible, since the hydrolysis reaction time no longer has to be limited to achieve an acceptable 7-ADCA-quality. Also during enzymatic synthesis no colour formation is observed any longer resulting in an improved quality of the β-lactam compound prepared. For instance, cefalexine with absorption of lower than 0.05, preferably lower than 0.04, more preferably lower than 0.03 has been prepared of course, the invention also

provides other ß-lactam with good colour, viz. low absorption at 400 nm.

Finally, as described in copending not prepublished International application no. EP 98/02458, an *N*-substituted ß-lactam can also be converted into another *N*-substituted ß-lactam by applying both a penicillin acylase and a dicarboxylase acylase, either apart or together.

In a preferred embodiment of the invention, a process is carried out without isolation and/or purification of any intermediates that may at one time or another be present in the reaction mixture. This way, no product is lost in an isolation or purification process.

The following examples are only presented to illustrate the invention.

Experimental methods for the examples 1-4

Turbidity measurement

Turbidity is expressed in EBC-units : a suspension of 5 g/L hydrazine sulphate and 50 g/L hexamethylene tetramine is used as a standard, i.e. yielding 1000 EBC units of turbidity as determined on an Unigalvo Nephelometer (EEL; Halstead,Essex; UK)

Colour measurement

Colour is expressed as the spectrophotometrically determined extinction at a wavelength of 425 nm, using a sample cell with an optical pathlength of 10 mm. In cases with reference to "colour" of solid PADCA or 7-ADCA, the figures refer to measurements on a solution of 2.0 grams of the respective solid preparation in 20.0 ml of 2N HCl.

Activity measurement

Activity is expressed in PADCA-units (one PADCA-unit is the amount of enzyme capable of converting one micromole of PADCA into 7-ADCA per minute under our standard conditions) and is measured titrimetrically by monitoring the initial-phase (i.c: the first 10 minutes) neutralization of the phenylacetic acid formed.

Assay conditions: 0.214 M PADCA; 35°C.; pH=7.85; neutralization by 0.1 N NaOH.

Stability measurement

Stability is expressed as the half life time, i.e. the time (in hours) elapsed after the enzyme activity has decreased to 50% of its original value under our standard conditions and with regular replacement of the reaction solution. Conditions are thereby chosen as close to the industrial relevant conditions as in practice possible.

Stability is determined by semi-continuous reuse of the enzyme sample as follows:
in a 100 ml thermostatted reaction vessel (equipped with pH-stat facilities and a sieve-bottom) about 2 grams of immobilized enzyme is introduced. In 4.1 hours cycles, 50 ml fresh substrate (0.214 M solution of PADCA, brought to pH = 7.85 by addition of a 3 M solution of ammonia) is added. To maintain the pH constant at pH = 7.85 a 3.0 M solution of ammonia is added, the addition rate is monitored; after 4 hours the reaction mixture is removed via the sieve bottom, leaving the immobilized enzyme inside the reactor to be reused in the next cycles. The ammonia addition rate during the first ten minutes of each cycle is denoted as the activity, expressed as PADCA-units. When the activity has decreased to about 50% of the mean acitivity of the first five cycles, the stability can be determined. Half life time is calculated by curve fitting the time dependence of the enzyme activity as a simple exponential function of the form:

$$At = AO*\exp(-t*\ln(2)/t1/2)$$

in which:

t : elapsed time in hours

AO,AT: enzyme activity (PADCA-units), mean value first five cycles and after t hours,respectively.

t1/2 : half life time (hours) as defined in text.

7-ADCA recovery

To 100 ml of a hydrolysed PADCA solution, concentrated sulphuric acid is added to realize a pH below 1.

Approximately 50 ml of a water-immiscible alcohol (e.g. isobutanol) is added and stirred for 10 minutes to allow extraction of the phenylacetic acid from the aqueous layer; after phase separation the aqueous phase is brought to pH 4 to 5 by the addition of a solution of 25% ammonia to crystallize 7-ADCA; the solidified 7-ADCA is filtered over a sintered glass filter, washed with approximately 50 ml of demineralized water and dried at 50 °C for about 16 hours until a dry substance percentage of > 97% is achieved. Yield is defined as total moles of 7-ADCA recovered per mole of PADCA in the reaction solution prior to acidification with sulphuric acid.

Example 1

To 800 ml of a fresh solution of 7 (w/w)% of PADCA in distilled water, a solution of 3 M ammonia is added until a pH of 7.5 is measured. This solution is referred to as: PADCA-solution A.

- 200 ml of the original PADCA solution A is filtered over Whatman GF/A paper (W&R Balston Ltd, UK) and the pH is adjusted to pH = 7.85 by addition of a solution of 3 M ammonia. This solution is referred to as A_1.

- To a second portion of the original PADCA solution A, 0.7 gram of active carbon (Norit SX Ultra, Norit BV, Amersfoort, Holland) is added. After 20 minutes gently stirring, the solution is filtered and the pH is adjusted to pH = 7.85 as in solution A_1. This solution is referred to as solution A_2.

- To a third portion of the original PADCA solution A, 0.1 gram of sodium metabisulphite is added. After 20 minutes gently stirring, the solution is filtered and the pH is adjusted as in solution A_1. This solution is referred to as solution A_3.

- To a fourth portion of the original PADCA solution A, 1 gram of sodium sulphite is added. The solution is

filtered and the pH is adjusted as in solution A_1. This solution is referred to as solution A_4.

- Turbidity and colour were measured from all four solutions. Samples of the four solutions were kept at room temperature and both turbidity and colour were measured at regular time intervals. The results are compiled in Tables 1.1 and 1.2. and plotted in Figures 1 and 2 respectively.

Table 1.1 Effects on colour of PADCA-solutions

Time (hours)	A_1	A_2	A_3	A_4
0.00	0.517	0.262	0.517	0.522
2.00	0.516	0.291	0.446	nd
3.25	0.530	0.296	0.435	nd
4.75	0.528	0.304	0.432	nd
6.00	0.524	0.319	0.437	nd
7.50	0.540	0.316	0.435	nd
25.25	0.579	0.356	0.381	0.333

Table 1.2 Effects on turbidity in PADCA-solutions

	Solution			
Time (hours)	A_1	A_2	A_3	A_4
0.00	1.0	0.4	1.0	1.0
1.00	1.4	0.8	0.7	nd
2.75	2.1	3.3	0.9	nd
4.25	4.7	3.6	0.7	nd
5.50	6.0	4.8	0.8	nd
7.00	6.1	5.8	0.8	nd
24.75	21.5	20.6	0.6	0.5

The enzyme activity of immobilized E.coli penicillin acylase (Sclavo/DeBi SpA, Siena, Italy) was determined (cf. Activity measurement). No significant differences were observed between the four figures.

Similar results were obtained with dithiothreitol or P-mercaptoethanol present as the reducing sulphur-compound. Non-sulphur reductors (ascorbic acid, nitrite, butylhydroxyanisol etcetera) did not dissolve the sulphur deposit, nor did sulphur-compounds such as formaldehyde sulphoxylate or thiosulphate.

Example 2

To each of a 100 ml sample of the PADCA solutions denoted A_1, A_2 and A_3 (cf. Example 1), approximately 4 grams immobilized E. coli penicillin acylase (Sclavo/DeBi SpA, Siena, Italy) were added. The hydrolysis was continued under addition of a solution of 3 M ammonia to maintain the pH at 7.85, for about 4 hours at 35°C; the enzyme was removed from each reaction solution. The solutions, obtained after hydrolysis of solutions A_1, A_2 and A_3 are referred to as B_1, B_2 and B_3 respectively.

Turbidity and colour were measured from all three solutions. Samples of the three solutions were kept at room temperature and both turbidity and colour were measured at regular time intervals. The results are compiled in Tables 2.1 and 2.2. and plotted in Figures 3 and 4, respectively.

Table 2.1 Effects on colour of 7-ADCA-solutions

	Solution		
Time (hours)	B_1	B_2	B_3
0.00	0.900	0.711	0.900
2.00	0.943	0.745	0.842
3.25	0.986	0.743	0.807
4.75	1.014	0.752	0.774
6.00	1.078	0.781	0.760

7.50	1.043	0.791	0.752
25.25	1.068	0.894	0.705

Table 2.2 Effects on turbidity in 7-ADCA-solutions

	Solution		
Time (hours)	B_1	B_2	B_3
0.00	21.0	48.0	21.0
1.00	21.5	48.0	12.0
2.75	22.0	49.0	4.0
4.25	23.5	52.0	2.3
5.50	26.0	57.0	1.8
7.00	26.0	52.0	1.4
24.75	33.0	77.0	0.6

Example 3

To a 200 ml portion of the original PADCA solution A (as described in Example 1), 0.2 gram of sodium metabisulphite is added. The solution is filtered and the pH is adjusted as in solution A_1. This solution is referred to as solution A_5.

To a 200 ml portion of the original PADCA solution A (as described in Example 1), 0.5 gram of sodium metabisulphite is added. The solution is filtered and the pH is adjusted as in solution A_1. This solution is referred to as solution A_6. The enzyme activity of immobilized E.coli penicillin acylase (Sclavo/DeBi SpA, Siena, Italy) was determined under close-to-application conditions over a period of 10-40 days (cf. Stability measurement) in solution A_1, A_2, A_3, A_5 and A_6. No significant difference was observed between the initial

activity figures. The results for the stability measurements are compiled in Table 3.1.

Table 3.1 Enzyme stability of immobilized E.coli penicillin acylase

Solution (hours)	half-life time
A_1	165
A_2	289
A_3	217
A_5	405
A_6	763

The effect of varying concentration of metabisulphite on the enzyme stability is plotted in Figure 5.

Example 4

To a 200 ml portion of the original PADCA solution A (as described in Example 1), 0.1 gram of sodium dithionite is added. The solution is filtered and the pH is adjusted as in solution A_1. This solution is referred to as solution A_7. To four 200 ml portions of solutions A_1, A_2, A_3, and A_4 (prepared as described in Example 1) and to the 200 ml of solution A_7, fresh portions of approximately 4 grams immobilized A.faecalis penicillin acylase were added; the hydrolysis was performed for about 4 hours at 35°C and addition of a solution of 3 M ammonia to maintain the pH at 7.85; the enzyme was removed and from each reaction solution the 7-ADCA was recovered as described in the paragraph "7-ADCA recovery". The recovered products from reaction mixtures A_1, A_2, A_3, A_4 and A_7 are referred to as C_1, C_2, C_3, C_4 and C_7, respectively. The results are compiled in Table 4.1.

Table 4.1 Colour and yield of 7-ADCA after recovery

Sample	Colour	Yield
C_1	0.295	96.1%
C_2	0.190	96.1%
C_3	0.157	96.2%
C_4	0.100	96.3%
C_7	0.168	96.0%

Example 5

Preparation of Cephalexin in the presence of sodium metabisulfite

In a typical procedure, enzymatic conversion of 7β-amino-3'-desacetoxycephalosporanic acid (7-ADCA) and phenylglycine amide into Cephalexin is carried out with recovery of excess 7-ADCA using electrodialysis. Recovered 7-ADCA is reused in the next cycle.

Preparation of an electrodialysate from an enzymatic Cephalexin synthesis

An enzymatic condensation reaction is carried out on a 2 liter scale using 7-ADCA (500 mM) and phenylglycine amide (500 mM) and the immobilized enzyme Assemblase® as described below ('enzymatic preparation of Cephalexin monohydrate'). At the end of the reaction the condensation effluent is separated from the immobilized enzyme. Crystalline Cephalexin monohydrate is obtained by shifting the pH to 7 using 25% sulfuric acid followed by filtration. The pH of the mother liquor is shifted to 4.5 using 25% sulfuric acid and crystalline 7-ADCA is formed and isolated by filtration. The remaining mother liquor contains ammonium sulfate (2.53%, w/w, based on sulfate), 7-ADCA (0.51%, w/w), Cephalexin (2.30%, w/w), phenylglycine (0.65%, w/w), and phenylglycine amide (1.29%, w/w).

At 5°C, the pH of the mother liquor is adjusted to 7 using 25% ammonia. The resulting solution is subjected to electrodialysis at 10-30 A using an 'Electrodialysepilotanlage Type BEA-I' (Berghof) equipped with AMX-SB and CMX-SB membranes (Tokoyama Soda) with a specific membrane surface of 9.4 m^2. Following electrodialysis, a desalted electrodialysate containing ammonium sulfate (0.33%, w/w, based on sulfate), 7-ADCA (0.45%, w/w), Cephalexin (2.00%, w/w), phenylglycine (0.56%, w/w), and phenylglycine amide (0.95%, w/w) is obtained. Together with the recovered crystalline 7-ADCA, this desalted electrodialysate is used in the next step.

Enzymatic preparation of Cephalexin monohydrate

A 3 liter feed vessel is charged with 7-ADCA (187.7 g; 876 mmoles; fresh), 7-ADCA (164.0 g; 766 mmoles; recovered as described above), phenylglycine amide (166.6 g), and electrodialysate (1292 g; prepared as described above, containing 5.81 g of 7-ADCA; 27 mmoles). After cooling to 5°C, the mixture is transferred to a 3 liter reactor equipped with a sieve-bottom and charged with Assemblase® (200 g, weighed after filtering and washing the beads with water). Assemblase® is an immobilised E-coli penicillin acylase from E-coli ATCC 11105 as described in WO 97/04086. The immobilisation has been carried out as described in EP-A-222462, applying gelatin and chitosan as gelling agent and glutaraldehyde as crosslinking agent. Immediately, the pH is adjusted to 7.7 using 25% ammonia. After stirring the mixture for 97 minutes at 5°C, the condensation effluent is separated from the immobilized enzyme by discharge through the sieve-bottom.

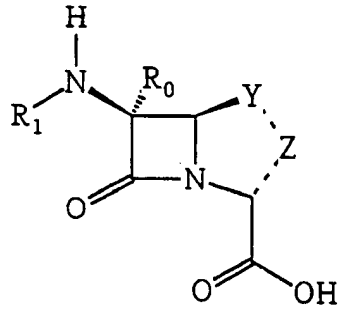
Cephalexin monohydrate is prepared from the condensation effluent by crystallization at pH 7.0 and 30°C using 25% sulfuric acid and a seed of 300 g of crystallization slurry from a previous batch. After cooling to 10°C, Cephalexin monohydrate crystals are obtained by centrifugation (leaving 300 g of the slurry behind to be used as seed in the next batch), washing of the crystals with water (2 x 300 g) and acetone (300 g), and drying.

Yield 179.3 g (491 mmoles, 29.4%). Quality: absorbance at 400 nm: 0.062.

In a comparative experiment using 7-ADCA (192.5 g; 899 mmoles; fresh), 7-ADCA (136.4 g; 637 mmoles; recovered as described above), phenylglycine amide (170.1 g), electrodialysate (1300 g; prepared as described above, containing 5.85 g of 7-ADCA; 27 mmoles), sodium metabisulfite (3.0 g) is added. Following work-up as described, 170.4 g of Cephalexin monohydrate is obtained (466 mmoles, yield 29.8%). Quality: absorbance at 400 nm: 0.030.

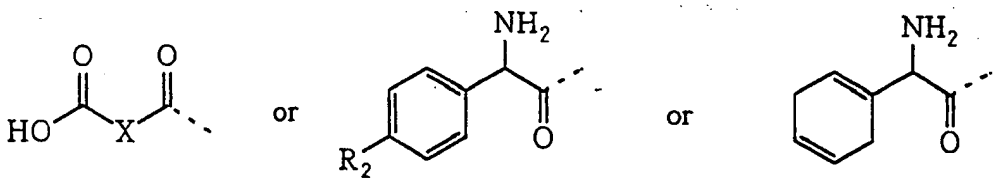
Claims

1. A method for preparing a β-lactam antibiotic having the general formula (I)



(I)

wherein R_1 is either hydrogen or



with

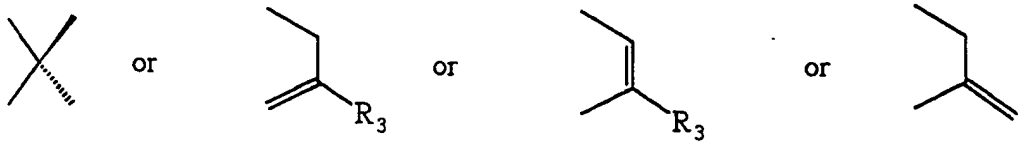
X is $(CH_2)_m$-A-$(CH_2)_n$, wherein m and n are the same or different and are chosen from the group of integers 0, 1, 2, 3 or 4, and A is CH=CH, C≡C, CHB, C=O, optionally substituted nitrogen, oxygen, sulphur or an optionally oxidized form of sulphur, and B is hydrogen, halogen, hydroxy, C_{1-3} alkoxy, or optionally substituted methyl, R_2 is hydrogen or hydroxy;

and wherein

R_0 is hydrogen or C_{1-3} alkoxy;

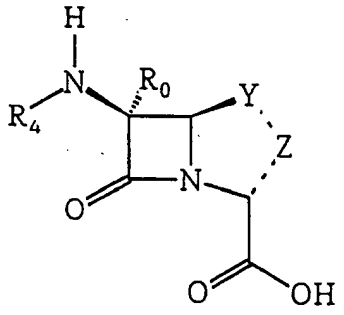
Y is CH_2, oxygen, sulphur, or an oxidized form of sulphur;

Z is



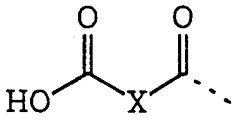
wherein R_3 is hydrogen, hydroxy, halogen, C_{1-3} alkoxy, optionally substituted, optionally containing one or more

heteroatoms, saturated or unsaturated, branched or straight C_{1-5} alkyl,
optionally substituted, optionally containing one or more heteroatoms, C_{5-8} cycloalkyl,
optionally substituted aryl or heteroaryl, or optionally substituted benzyl,
or a salt thereof,
by reacting a compound with the formula I'

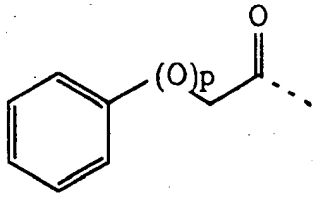


(I')

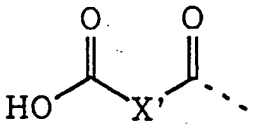
with R_4 is either



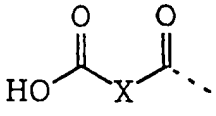
or



with X as defined above, and p = 0 or 1,
when R_1 is hydrogen, and each of R_0, Y and Z have corresponding meanings in β-lactams with formula I and I', with a dicarboxylate acylase, or a penicillin acylase respectively;
or when R_4 is H, or



with X' is defined as X but different from the same when R_1 is



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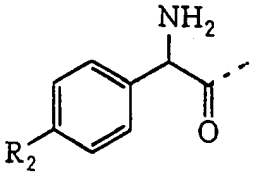

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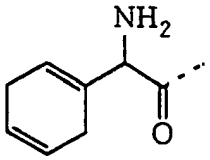
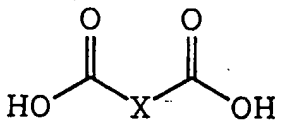


respectively, and each of R_0, Y and Z have corresponding meanings in β-lactams with formula I and I',
with a precursor for a side chain of the formula



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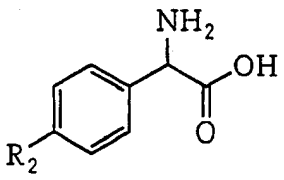
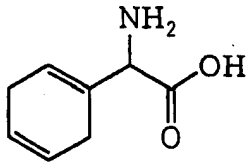


or



respectively, or an amide or ester thereof
and a dicarboxylate acylase or a penicillin acylase, respectively if R_4 is hydrogen and a penicillin acylase and dicarboxylate if R_4 is not hydrogen,
characterized by the application of a sulphur reducing compound.

2. A process according to claim 1, characterized by the application of a sulphur containing compound selected from the group consisting of sulphite, metabisulphite, dithiothreitol, β-mercapto-ethanol and dithionite.

3. A process according to claim 2, characterized by the application of the sulphur containing compound in a weight ratio of 0.02 - 1% compared to the reaction mixture.

4. Cefalexine with an absorption of lower than 0.050, preferably lower than 0.040, more preferably lower than 0.030 at 400 nm.

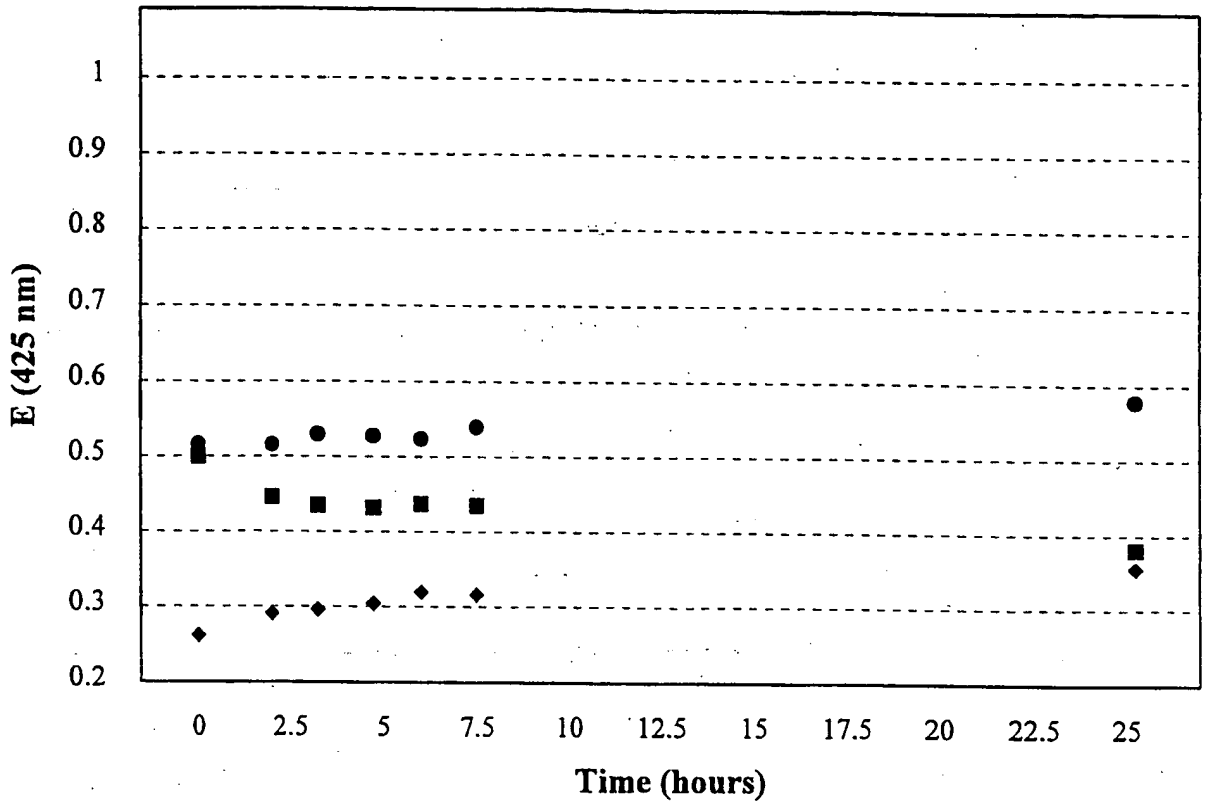


Figure 1

blank
with activated carbon
with sodium metabisulphite

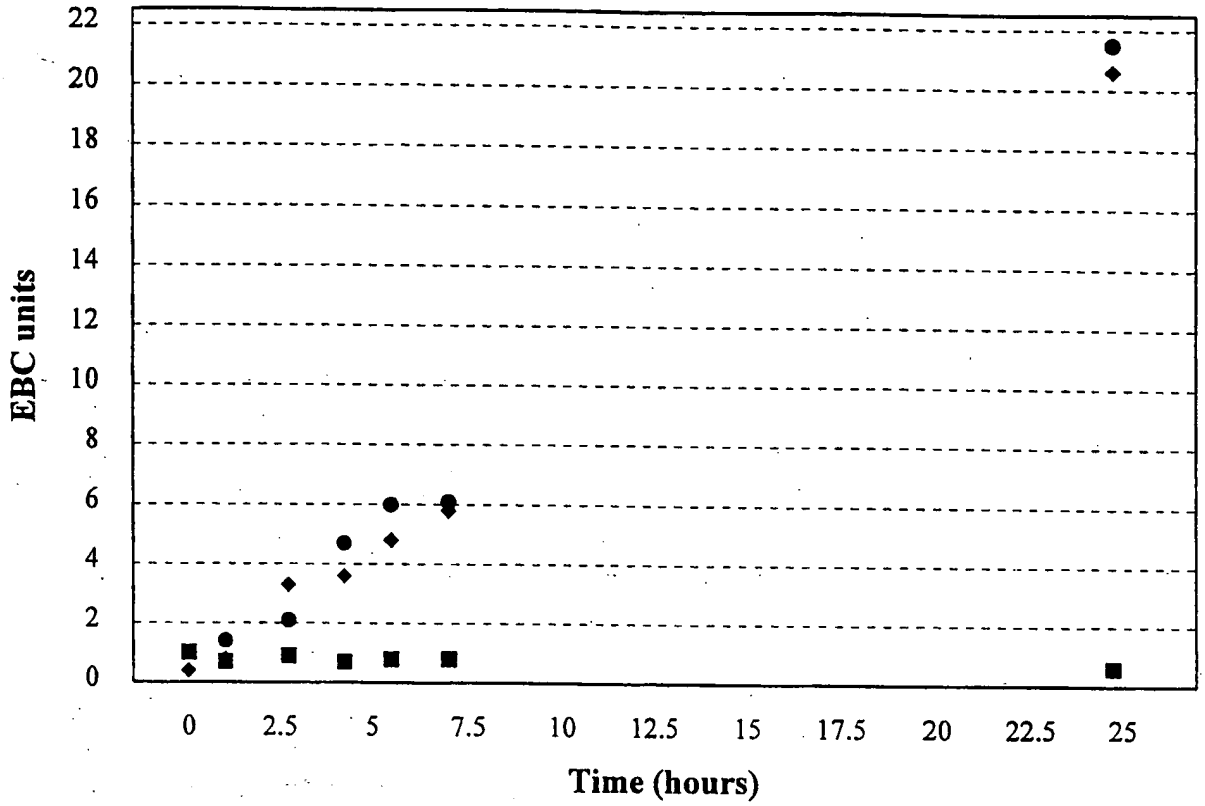


Figure 2

- blank
- with activated carbon
- with sodium metabisulphite

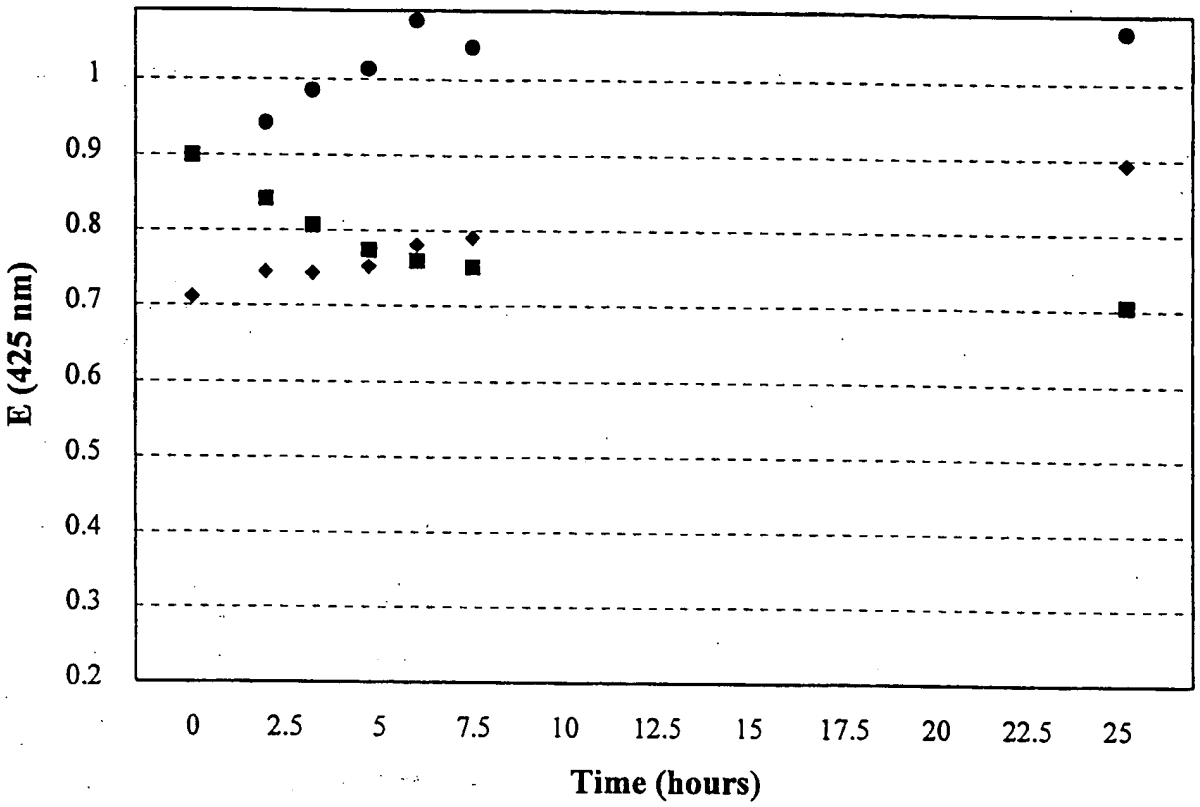


Figure 3

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- with activated carbon
- with sodium metabisulphite

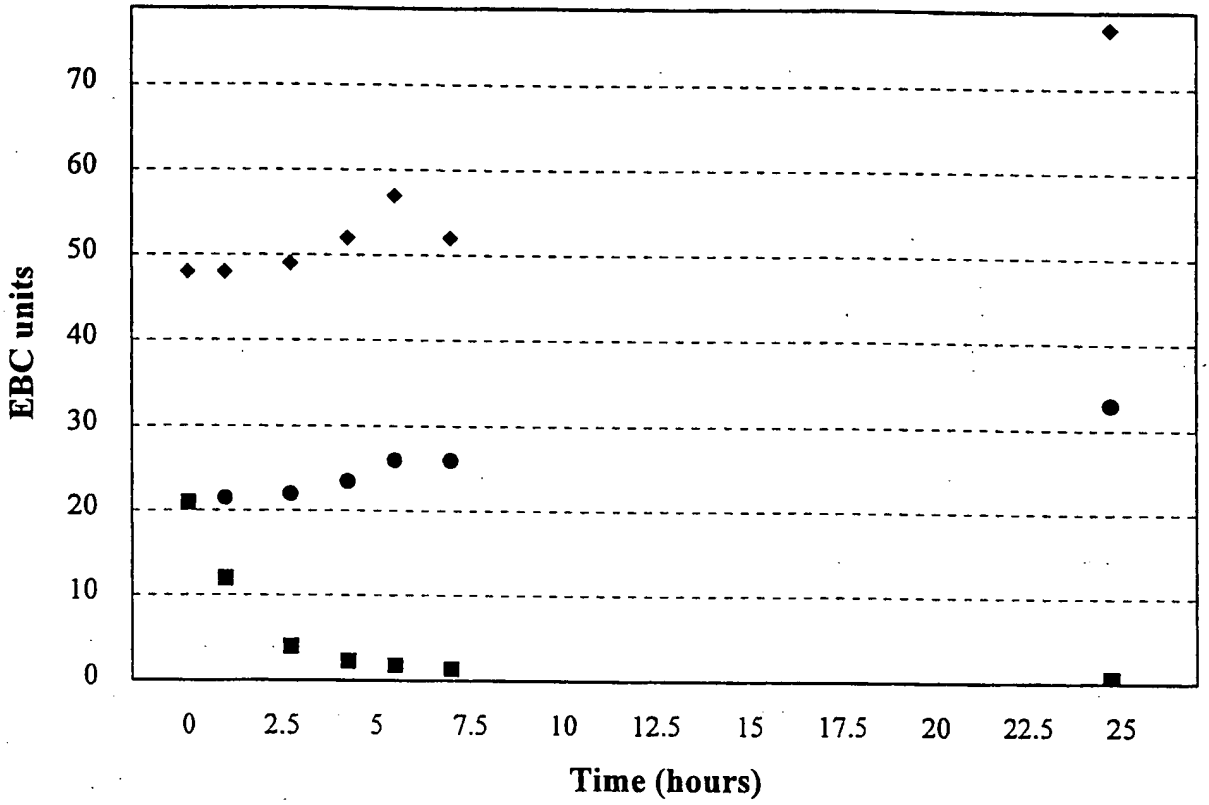

Figure 4

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- with activated carbon
- with sodium metabisulphite

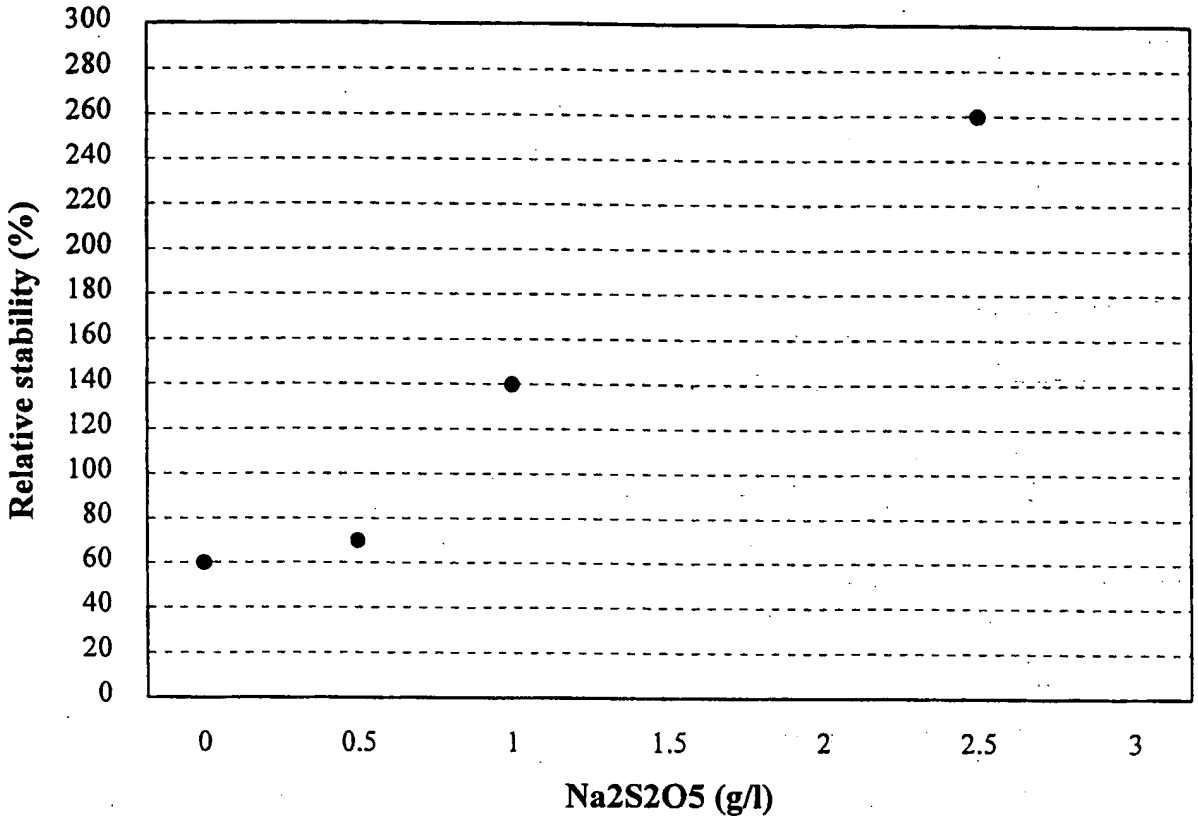
Relative stability (%)
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
Na2S2O5 (g/l)

Figure 5

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 98/03628

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12P35/04 C12P37/04 C12P17/10 C12P17/18

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P C12N C07C

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and. where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document. with indication, where appropriate. of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 115, no. 1, 8 July 1991 Columbus, Ohio, US; abstract no. 4121, ERARSLAN, ALTAN ET AL: "Kinetic investigation of penicillin G acylase from a mutant strain of Escherichia coli ATCC 11105 immobilized on oxirane-acrylic beads" XP002078718 see abstract & J. CHEM. TECHNOL. BIOTECHNOL. (1991), 51(2), 181-95 CODEN: JCTBED;ISSN: 0268-2575, ---	1
Y	FR 2 228 783 A (GLAXO LAB LTD) 6 December 1974 see page 4; claims ---	1
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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